

Inhibition of Platelet Aggregation by Supernates from Stored Red Blood Cells

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ABSTRACT

Uncontrolled hemorrhage is the second leading cause of death to war fighters on the battlefield next to central nervous system injury. Hemorrhagic deaths are potentially preventable if bleeding can be controlled early, but hemorrhage control is complicated by trauma-induced coagulopathy characterized by loss of platelets and coagulation factors, dysfunctional platelets, and increased fibrinolysis. The specific dysfunction of platelets remains to be elucidated, but may be of greater importance than platelet number for clot integrity. In a previous study of the proinflammatory effects of supernates from stored RBC, we observed that platelets in blood incubated with supernates from stored RBC exhibited lower anti-CD41a-FITC fluorescence, which indicated decreased expression of GPIIb/IIIa on the platelet membrane. Because activated GPIIb/IIIa binds fibrinogen and facilitates formation of platelet aggregates that are necessary to form stable platelet plugs during hemostasis, we designed this study to determine if supernates from RBC impaired platelet aggregation as a consequence of reduction in GPIIb/IIIa expression. Blood was collected from healthy volunteers, prepared into non-leukoreduced (NLR) and leukoreduced (LR) packed RBC with AS-5 and stored at 1-6°C for six weeks. Supernates obtained from samples collected from RBC units every two weeks were mixed with freshly collected ABO-compatible blood. Platelets in each incubated blood sample were evaluated for GPIIb/IIIa expression by flow cytometry and for aggregation response to collagen by whole blood aggregometry. We observed that supernates from stored RBC decreased GPIIb/IIIa expression and inhibited platelet aggregation proportionately. Furthermore, both activities increased as a function of storage age, which was indicative of accumulation of the bioactive substance(s). Supernates from LR RBC, however, did not alter either GPIIb/IIIa or aggregation. We also showed that the inhibitor that accumulates during storage interacts directly with platelets rather than stimulates accessory cells to release an inhibitor. Characterization studies indicated that the inhibitor is not lipid, but is hydrophilic, is resistant to protease, and is greater than 30 kD molecular weight. We discuss the potential impact transfusion of this inhibitor may have on controlling hemorrhage.

1.0 INTRODUCTION

Massive transfusion, defined as greater than ten units of RBC transfused within a twenty-four hour period, is more common in military casualties than in civilian trauma.[1] Whereas, 1-3% of civilian trauma requires massive transfusion, 8% of casualties that occurred in the war in Iraq up to 2007 required massive transfusion and up to 16% of casualties in the Vietnam war were estimated to require massive transfusion.[1] Transfusion of large numbers of units of RBC results from uncontrolled hemorrhage, which is a leading cause of death to war fighters on the battlefield.[2-4] Potentially, these deaths are preventable if hemorrhage can be controlled early.[1, 3, 5, 6] Trauma, however, induces a coagulopathic state characterized by loss of coagulation factors and platelets through bleeding and consumption, dysfunction of platelets and the coagulation system, and increased fibrinolysis.[4, 7, 8] In addition,

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14. ABSTRACT

Uncontrolled hemorrhage is the second leading cause of death to war fighters on the battlefield next to central nervous system injury. Hemorrhagic deaths are potentially preventable if bleeding can be controlled early, but hemorrhage control is complicated by trauma-induced coagulopathy characterized by loss of platelets and coagulation factors, dysfunctional platelets, and increased fibrinolysis. The specific dysfunction of platelets remains to be elucidated, but may be of greater importance than platelet number for clot integrity. In a previous study of the proinflammatory effects of supernates from stored RBC, we observed that platelets in blood incubated with supernates from stored RBC exhibited lower anti-CD41a-FITC fluorescence, which indicated decreased expression of GPIIb/IIIa on the platelet membrane. Because activated GPIIb/IIIa binds fibrinogen and facilitates formation of platelet aggregates that are necessary to form stable platelet plugs during hemostasis, we designed this study to determine if supernates from RBC impaired platelet aggregation as a consequence of reduction in GPIIb/IIIa expression. Blood was collected from healthy volunteers, prepared into non-leukoreduced (NLR) and leukoreduced (LR) packed RBC with AS-5 and stored at 1-6°C for six weeks. Supernates obtained from samples collected from RBC units every two weeks were mixed with freshly collected ABO-compatible blood. Platelets in each incubated blood sample were evaluated for GPIIb/IIIa expression by flow cytometry and for aggregation response to collagen by whole blood aggregometry. We observed that supernates from stored RBC decreased GPIIb/IIIa expression and inhibited platelet aggregation proportionately. Furthermore, both activities increased as a function of storage age, which was indicative of accumulation of the bioactive substance(s). Supernates from LR RBC, however, did not alter either GPIIb/IIIa or aggregation. We also showed that the inhibitor that accumulates during storage interacts directly with platelets rather than stimulates accessory cells to release an inhibitor. Characterization studies indicated that the inhibitor is not lipid, but is hydrophilic, is resistant to protease, and is greater than 30 kD molecular weight. We discuss the potential impact transfusion of this inhibitor may have on controlling hemorrhage.

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anticoagulant and preservative solutions in transfused blood products dilute factors and platelets to even lower concentrations.[1, 4, 9] Hemorrhage control is difficult to achieve with these conditions.

In addition to the dilutional impact of transfused blood products, it is not known if transfused blood products exert a direct effect on platelet function. It is well documented, however, that the plasma fraction of RBC (supernate) accumulate bioactive substances during storage that exhibit proinflammatory activities in blood *in vitro*. In earlier studies, we demonstrated that RBC stored in AS-5 solution accumulate substances that increase expression of Mac-1 on neutrophils (PMNs) and increase formation of PMN-platelet aggregates in fresh whole blood.[10] In those experiments, we observed that platelets in blood incubated with supernates from stored RBC displayed decreased fluorescence from FITC-conjugated antibody to CD41a (GPIIb/IIIa), which suggested that expression of GPIIb/IIIa on the platelet membrane was reduced. Because of the critical role of GPIIb/IIIa in platelet aggregation, we asked if the observed decrease in CD41a-FITC fluorescence correlated with decreased platelet aggregation activity. In this paper, we present results from two studies whereby the effects of supernates from stored RBC on platelet aggregation were determined and characterized. The first study demonstrates that RBC supernates accumulate during storage a substance or substances that reduce expression of GPIIb/IIIa on platelet membranes and impair platelet aggregation in the presence of a physiological agonist. This study has recently been published online in *Transfusion* early view and figures 1-4 of this manuscript are reprinted from that article.[11] In the second study we present results of our characterization of this inhibitor.

2.0 METHODS

2.1 Blood Collection and Processing

One blood collection was performed for each study. Collection and preparation of blood into AS-5 RBC was the same for both collections except for the number of units collected and the leukocyte status. The first protocol was described in detail previously.[10] Briefly, 450 ml of blood was collected from each of nineteen type A and type O volunteer donors in the first collection and from each of five type A donors in the second collection into Teruflex Blood Bag Systems (Terumo Corporation, Tokyo, Japan) that contained CPD anticoagulant in the primary collection bag. An institute review board approved the blood collection protocols before volunteers were recruited. All donors met AABB criteria for blood donation. In the first collection protocol, each donor was assigned randomly to either non-leukoreduced (NLR) or leukoreduced (LR) treatment arms, and LR-assigned units were passed through Leukotrap-SC RC Filtration Systems (Pall, East Hills, NY). None of the RBC units obtained in the second collection were leukoreduced. All units were then centrifuged at 4,100 x g, 4°C for 5 minutes without application of the brake. Plasma was expressed from each unit and 100 ml of AS-5 was added from a satellite bag to the packed cells. Sampling ports that consisted of 7 inches of sterile tubing connected to a sterile female Luer-Lok fitting were connected to each RBC unit using a sterile connecting device (SCD 312 Sterile Tubing Welder, Terumo Medical Corporation, Elkton, MD). All units were stored at 1-6°C for 42 days.

Twenty-five milliliter aliquots of RBC were collected aseptically from each unit of both collections on days 0, 14, 28, and 42 of storage, and centrifuged twice at 2,000 x g for 10 min at 5°C to obtain supernates. Supernates were stored as 0.5-1.5 ml volumes in sterile cryovials, and the vials were stored at -80°C. For each experiment, aliquots were thawed quickly in a 37°C water bath just before use.

2.2 Study I

2.2.1 GPIIb/IIIa Expression

Supernates were mixed with type-matched whole blood collected from three donors of each blood type. The final ratio of whole blood to supernate was 2:1. For each experiment, blood was collected first into one red top Vacutainer tube (no anti-coagulant) and discarded followed by collection into 3.2% sodium

citrate Vacutainer tubes. Within fifteen minutes of collection, 200 μ l of type-matched blood was added to each tube that contained 100 μ l of supernate and to two tubes that contained 100 μ l of Tyrode's buffer (one for each blood type) that acted as the negative control. Positive control tubes contained 15 μ l of thrombin (1 unit final concentration; Sigma-Aldrich, Saint Louis, MO), 30 μ l of H-Gly-Pro-Arg-Pro-OH (GPRP; 2.5 mM final concentration; Calbiochem, San Diego, CA) and 255 μ l of whole blood. All tubes were incubated in a 37°C water bath for thirty minutes, and transferred to ice.

Then, 50 μ l of each supernate/blood mixture and control mixtures was incubated with 15 μ l anti-CD41a-FITC antibody (clone HIP-8, BD Pharmingen, San Jose, CA) in sterile 12 x 75 mm polypropylene tubes for 30 min on ice. After incubation, RBC were lysed and leukocytes and platelets were fixed by incubation with 1 ml of 1X FACSLYSE (BD Biosciences, San Jose, CA) for ten min at room temperature. Cells were washed once with 450 μ l of Tyrodes buffer after centrifugation at 2,000 x g for 5 minutes. After removal of the wash fluid, blood cells were analyzed on a BD Biosciences FACSsort flow cytometer utilizing log forward scatter (FSC) and side scatter (SSC) amplifiers. Forward scatter versus CD41a-FITC fluorescence (FL1 channel) was used to gate the platelet population, and GPIIb/IIIa expression on platelets was quantified as geometric mean CD41a-FITC fluorescence by histogram analysis.

2.2.2 Aggregation Assay

Blood was collected from healthy volunteers into 3.2% sodium citrate Vacutainer tubes after collection into one red-top tube, which was discarded. The blood was kept on a rotator at 37°C during each experiment. Whole blood aggregometry was used to assess the aggregation response to 5 μ M collagen. Blood (500 μ l) was incubated for 30 min at 37°C with 250 μ l of either Tyrode's buffer (control) or supernates derived from RBC stored for 0 and 42 days from the first blood collection. Following incubation, the magnitude (amplitude) and time before onset of platelet aggregation (lag) in each mixture was measured by impedance in a Chrono-Log Model 700 aggregometer (Havertown, PA).

2.3 Study II

2.3.1 Aggregation Assay

Assay conditions with samples from the second blood collection differed slightly from those used in the first study. Either 450 μ l of blood or 450 μ l of platelet rich plasma (PRP) was mixed with 225 μ l of supernate plus 225 μ l of Tyrode's buffer and incubated for ten minutes at 37°C. Supernates from RBC stored for 0, 14, 28, and 42 days were assessed. In control assays, 450 μ l of blood were mixed with 450 μ l of Tyrode's buffer and incubated for ten min. Following incubation, the magnitude and lag time before aggregation for each mixture was measured as done in study I.

2.3.2 Characterization of Aggregation Inhibiting Substance

The inhibiting substance(s) in supernate from stored RBC was characterized with regard to dose response, chemical nature, and size. To assess dosage effects, supernate volumes in the aggregation assay were decreased and replaced with Tyrode's buffer to produce specified dilutions. To characterize the chemical nature of the inhibitor, supernates were treated with protease (5 units/ml from *Streptomyces griseus*, 24 hours, 37°C) in one experiment and in a separate experiment were extracted with a chloroform:methanol (1:2) mixture according to Bligh and Dyer.[12] For each 1 ml of supernate, 3.75 ml of chloroform:methanol (1:2) were added and vortexed. Then 1.25 ml CHCl_3 was added and the mixture was vortexed. This was followed by addition of 1.25 ml of distilled water and vortexing. The mixtures were centrifuged at 200 x g for 5 min at room temperature to separate the phases. Each phase was removed carefully and transferred to clean tubes. The tubes were placed into warm water and the phases were evaporated to dryness by a nitrogen stream. Solid material that had formed at the organic/aqueous interface was dried similarly. Dried phases were redissolved into the original volume of 5% albumin in

water for aggregation inhibition assays. The solid that formed during the extraction was dissolved into water. Aggregation inhibition assays were carried out as described above. Characterization of the size of the inhibitor was done by fractionation using specific molecular weight cutoff membranes. Supernates from two stored units were centrifuged through 30 kilo Dalton (kD) molecular weight cut off membranes. The retentate (>30 kD) was saved for testing and the filtrate was centrifuged through 10 kD membranes. The retentate (10- 30 kD) and filtrate (<10 kD) were saved for testing.

2.3.3 Statistical Analyses

Analysis of variance was used to test for differences among all treatment groups. Then the Bonferroni t-test was used for multiple comparisons to determine if the null hypothesis of no difference between two groups could be rejected.

3.0 RESULTS

3.1 GPIIb/IIIa Expression

Mean CD41a-FITC fluorescence of platelets decreased in blood incubated with supernates from stored NLR RBC (Figure 1). Reduction of CD41a-FITC fluorescence indicated that expression of GPIIb/IIIa on platelet membranes was reduced. An inverse relationship was observed between GPIIb/IIIa expression in the presence of supernates and the storage age of the RBC from which the supernates were derived. Platelet expression of GPIIb/IIIa was reduced by 15%, 25%, and 31% in blood incubated with supernates from NLR RBC stored for fourteen, twenty-eight, and forty-two days, respectively as compared to blood incubated with Tyrode's buffer. Supernates from LR RBC, however, did not affect GPIIb/IIIa expression. Supernates obtained on the day of blood collection (day 0) from either NLR or LR RBC did not alter GPIIb/IIIa expression from that observed on platelets of blood incubated with Tyrode's buffer.

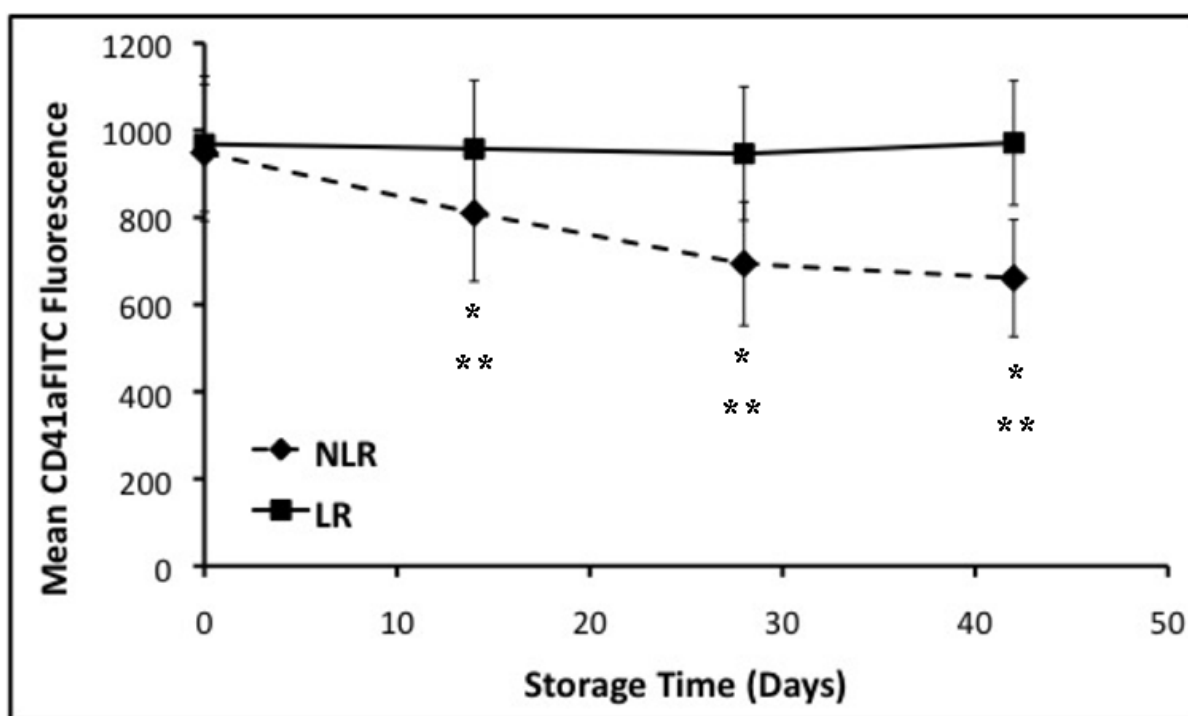


Figure 1: Effect of AS-5 Supernates on Platelet GPIIb/IIIa Expression as a Function of Storage Time.

Supernates from non-leukoreduced (NLR) and leukoreduced (LR) RBC collected at various storage times were incubated with whole blood and expression of platelet GPIIb/IIIa was assessed as described in “METHODS.” Mean CD41a-Fitc fluorescence represents platelet GPIIb/IIIa expression. Values are presented as Means \pm SD (n = 30 [Day 28 n = 25], NLR; n = 27 [Day 28 n = 23], LR). t-test comparisons are: *Day n NLR vs Day 0 NLR (p<0.003); **NLR vs LR (p<0.002).

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3.2 Platelet Aggregation

Figure 2 shows mean aggregation data acquired from mixing supernates from ten stored RBC units with blood from three donors (n = 30). The mean extent of platelet aggregation (amplitude) in blood incubated with supernates from NLR RBC stored in AS-5 for forty-two days was 31% lower than the mean extent of platelet aggregation in blood incubated with supernates from RBC stored for 0 days (day of collection). Coincident with decreased aggregation amplitude, supernates from NLR RBC stored for forty-two days also delayed the time before onset of aggregation (lag time). The mean lag time increased by 21%. Lag time was more variable than amplitude, but correlated with changes in amplitude (Figure 3). Supernates from RBC that had been leukoreduced before storage did not exert a statistically significant effect on either the time of onset or extent of aggregation.

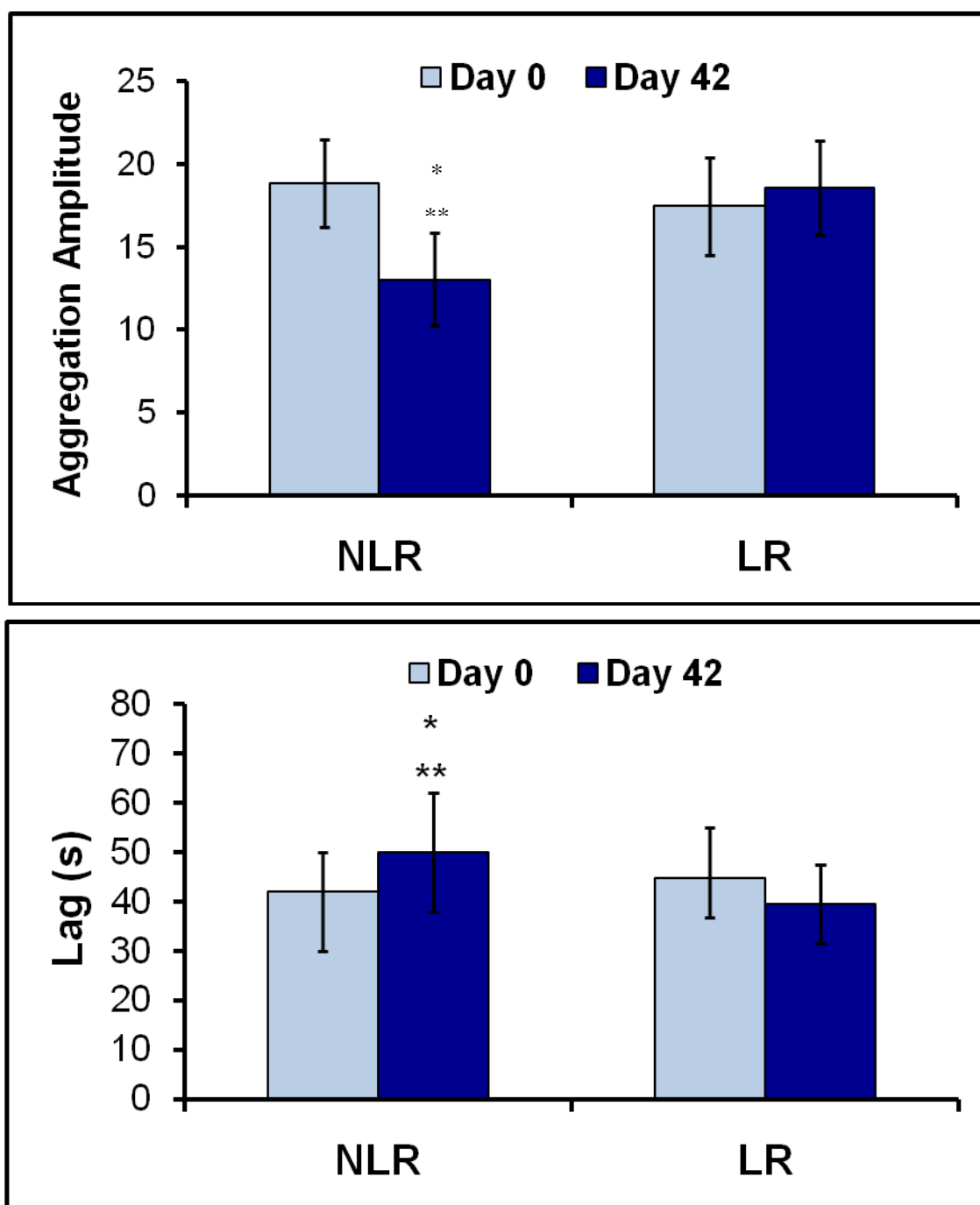


Figure 2: Effect of AS-5 Supernates on Platelet Aggregation.

Amplitude represents the magnitude of the platelet aggregation response as measured by changes in impedance and lag represents the time (s) until onset of aggregation. Treatment groups are: non-leukoreduced (NLR) and leukoreduced (LR). Values are presented as Means \pm SD (n = 30, NLR; n = 27, LR). t-test comparisons are: *Day 42 NLR vs Day 0 NLR (p<0.001); **Day 42 NLR vs Day 42 LR (p<0.001).

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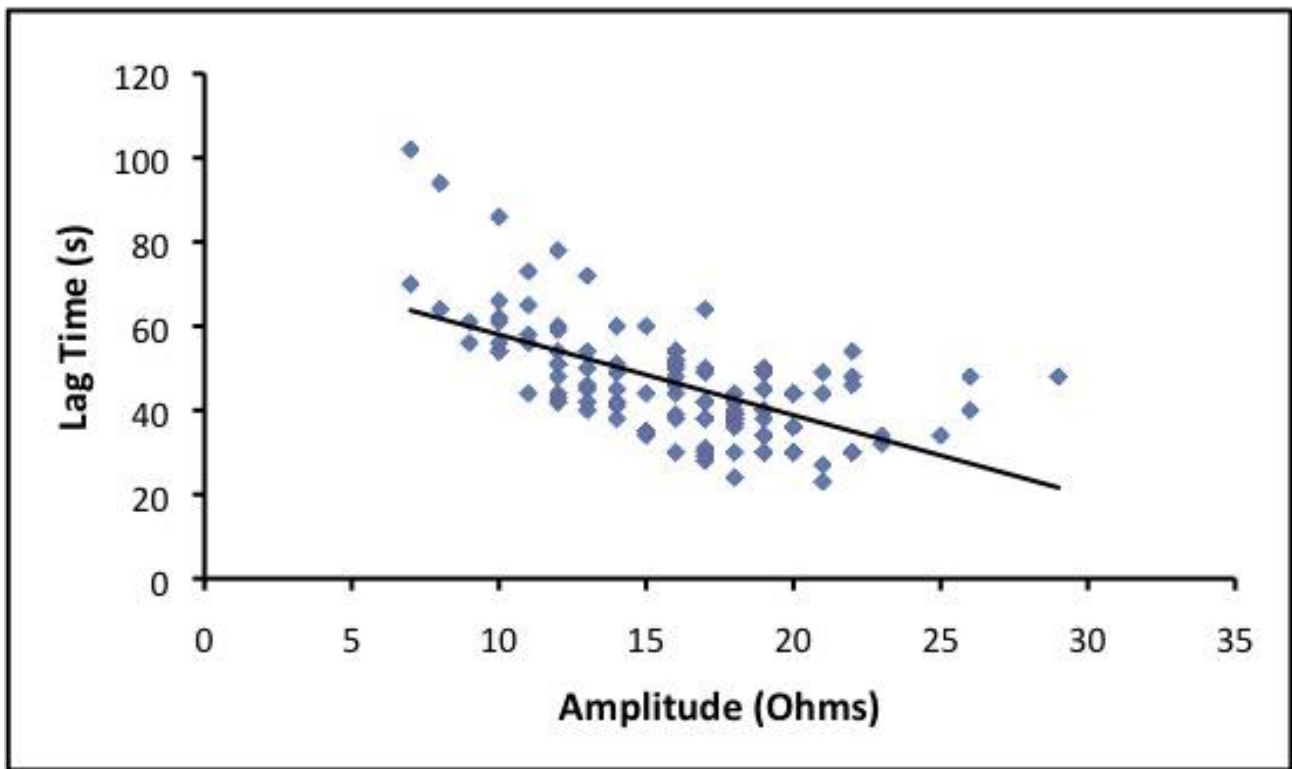


Figure 3: Correlation Between Lag Time and Aggregation Amplitude.

Graph shows amplitude and lag time data for NLR day 0 and day 42 supernate treatments. The equation of the regression line is: $y = -1.9x + 77.2$, $R^2 = 0.37$.

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Analysis of aggregation data from all samples—blood incubated with Tyrode’s buffer, and supernates from NLR and LR RBC—with respect to mean CD41a-FITC fluorescence revealed a correlation between the magnitude of aggregation (Amplitude) and the level of GPIIb/IIIa expression on platelets (mean CD41a-Fitc Fluorescence), and an inverse correlation between lag time and GPIIb/IIIa expression (Figure 4).

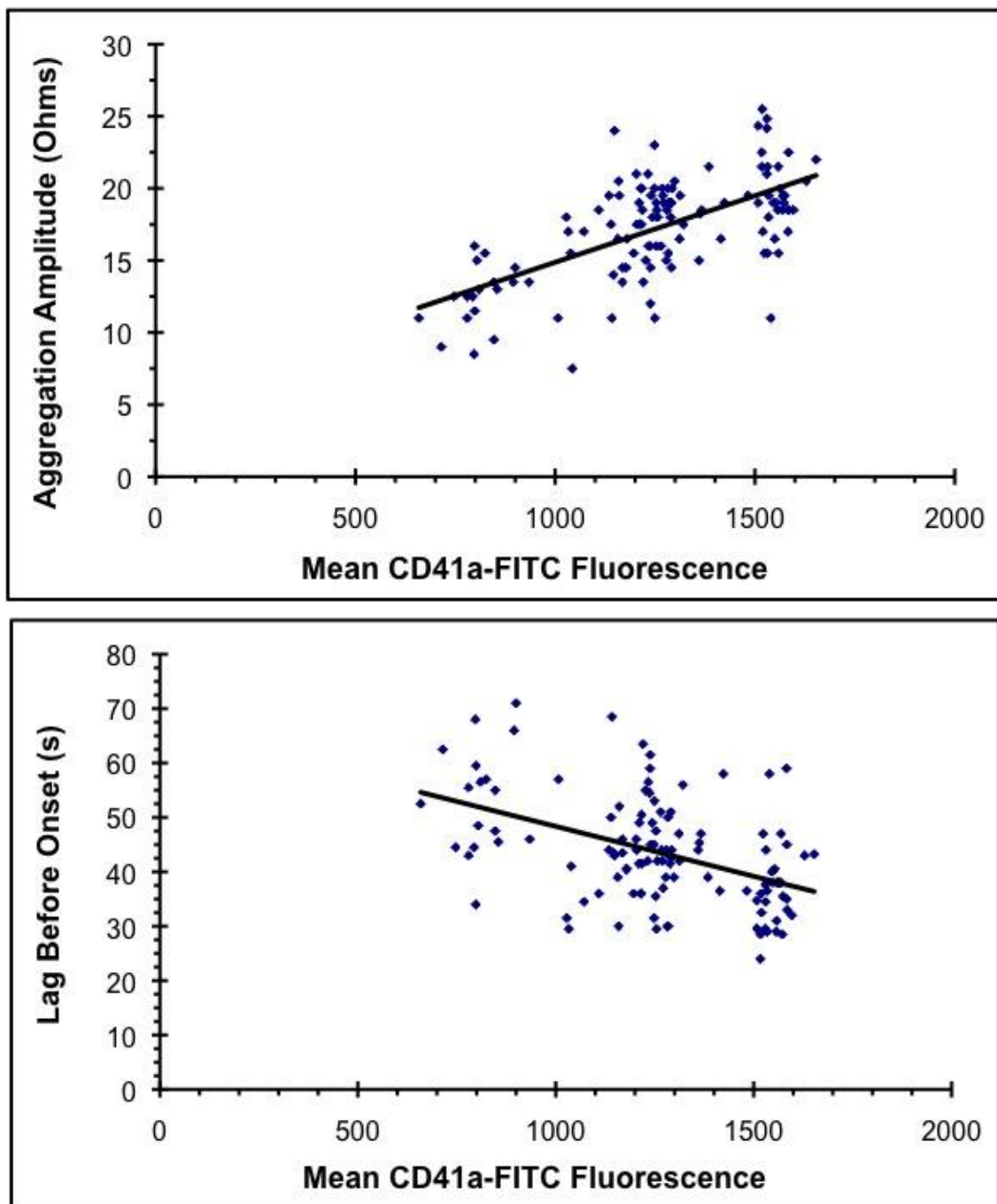


Figure 4: Relationship Between Aggregation Variables and GPIIb/IIIa Expression.

Amplitude represents the magnitude of the platelet aggregation response as measured by changes in impedance and “Lag” represents the time before the onset of aggregation after addition of collagen. Mean CD41a-FITC fluorescence represents platelet GPIIb/IIIa expression. Treatment groups are: non-leukoreduced (NLR) and leukoreduced (LR). Values are presented as Means \pm SD (n = 30, NLR; n = 27, LR).

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3.3 Characterization Study

Initially, we assessed the effect of storage time on the level of platelet aggregation inhibitory activity and observed that the level of platelet aggregation inhibitory activity in supernates increased as a function of the storage age of the RBC (Figure 5). Significant inhibitory activity was observed with supernates collected from RBC at fourteen days, the earliest storage time of our study. The mean percent inhibition of platelet aggregation in whole blood by supernates from NLR RBC stored for fourteen, twenty-eight, and forty-two days was 23%, 28% and 54% respectively. The nearly two-fold increase in inhibitory activity in supernates from day 28 to supernates from day 42 was highly significant ($p < 0.0001$).

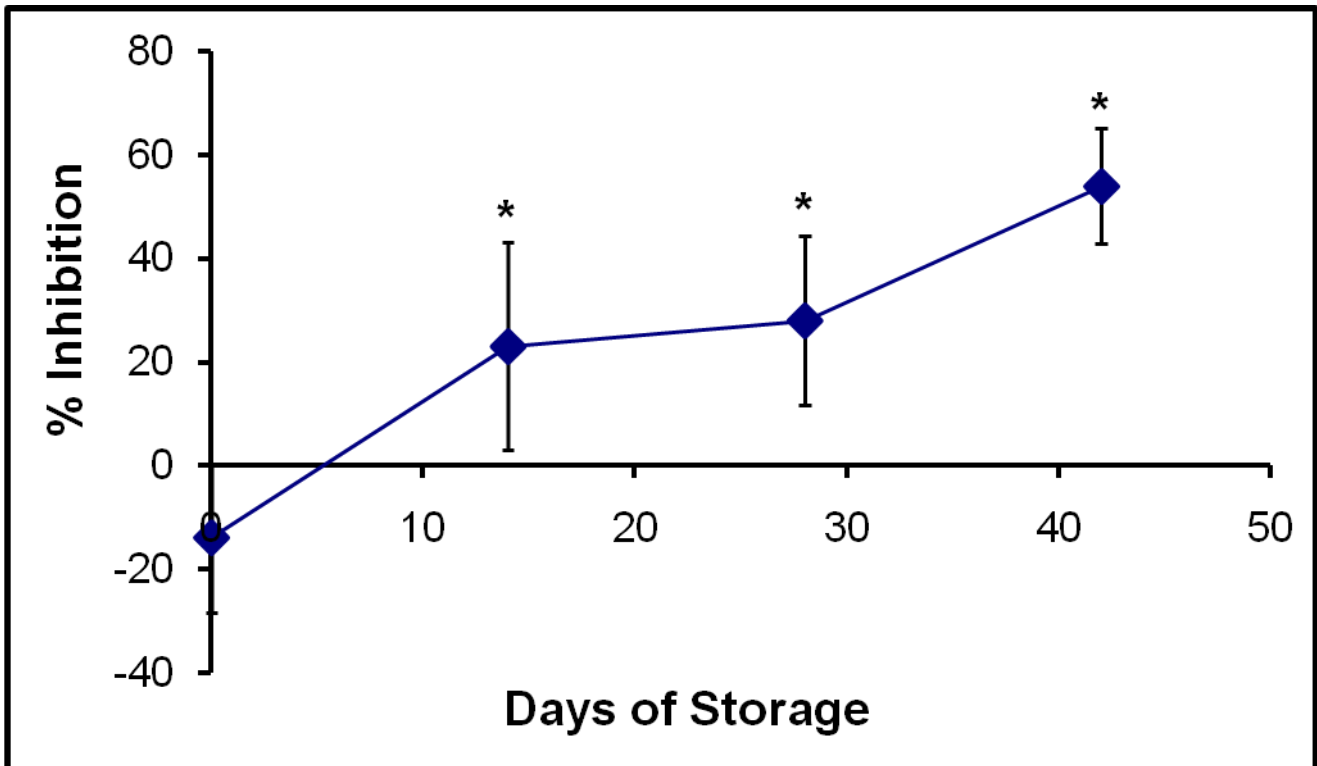


Figure 5: Platelet Aggregation Inhibition as a Function of RBC Storage Age.

Aggregation response to 5 μ M collagen was measured in whole blood mixed with supernates from NLR RBC collected at specified storage times according to “METHODS.” “% Inhibition” represents the percent decrease in the amplitude of platelet aggregation response in blood mixed with supernates compared to in blood mixed with Tyrode’s buffer (* $p < 0.003$, $n = 12$ for each day).

Figure 6 shows that dilution of day 42 supernates with Tyrode’s buffer resulted in decreased inhibition of platelet aggregation in a dose response. Platelet aggregation was inhibited by 57%, 38%, and 17% in the presence of 2-, 4-, and 8-fold diluted day 42 supernate, respectively. Variability in the inhibitory effect increased with increasing dilution consistent with variable levels of inhibitor accumulation in stored RBC units from different donors.



Figure 6: Dose Response of Platelet Aggregation Inhibitory Activity in Day 42 Supernates.

Aggregation response to 5 μ M collagen was measured in whole blood mixed with varying dilutions of supernates from NLR RBC stored for 42 days according to "METHODS." "% Inhibition" represents the percent decrease in the amplitude of platelet aggregation response in blood mixed with supernates compared to in blood mixed with Tyrode's buffer (* $p < 0.001$ vs Tyrode's buffer, $n = 8$).

Our whole blood assay system contained all blood cells. Therefore, it was not clear as to the mode of action of the inhibitor(s), that is, if the active agent(s) in RBC supernate acts directly on the platelet or interacts with another cell type to cause release of an inhibitor of aggregation. To address this question we compared the inhibition of platelet aggregation measured in whole blood and in PRP by day 42 supernates. Figure 7 shows that there was no significant difference in the observed inhibitory activity.

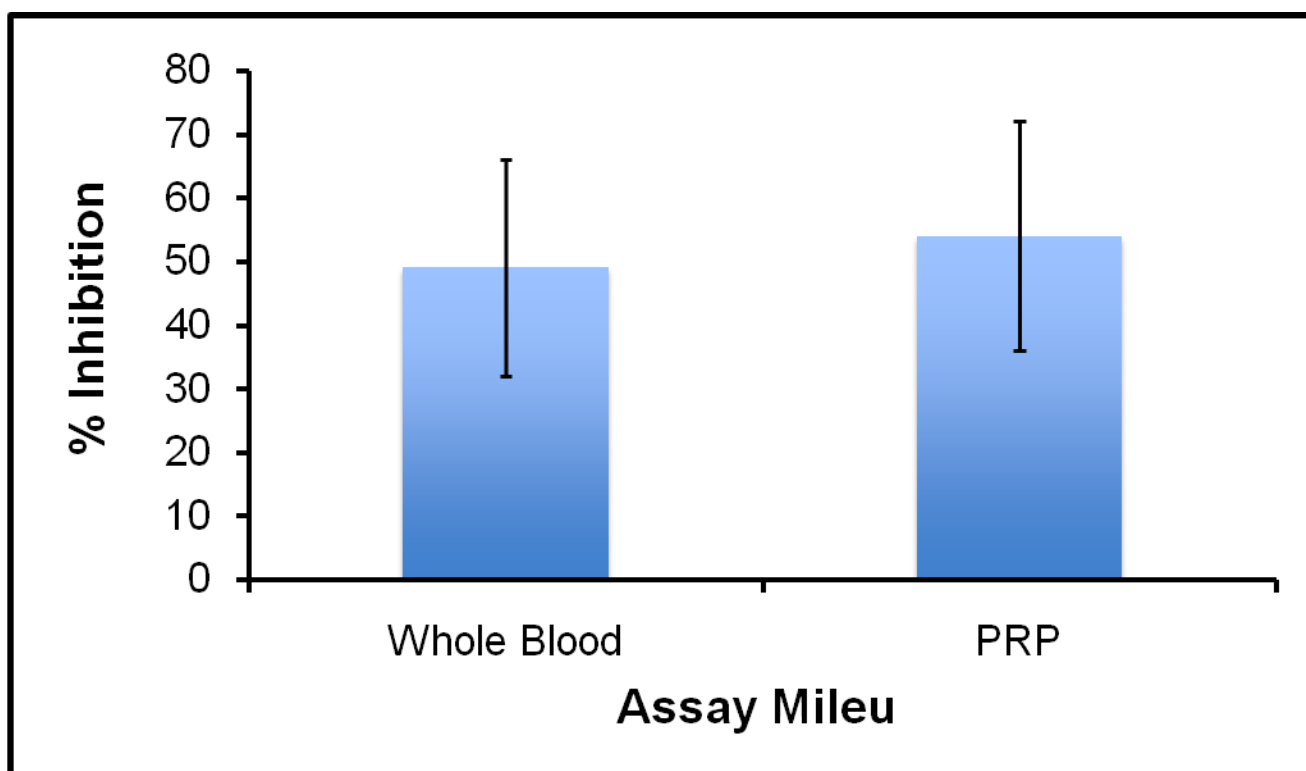


Figure 7. Inhibition of Platelet Aggregation in Whole Blood and PRP

Aggregation response to 5 μ M collagen was measured in whole blood and PRP mixed with supernates from NLR RBC stored for 42 days according to “METHODS.” “% Inhibition” represents the percent decrease in the amplitude of platelet aggregation response in blood mixed with supernates compared to in blood mixed with Tyrode’s buffer.

Figure 8 summarizes the effects of protease treatment, extraction with chloroform:methanol, and size fractionation. “Pre” and “Post” samples show the level of inhibitor activity in the supernates before and after treatment, respectively. Overnight treatment of supernates with protease from *Streptomyces griseus* did not alter the platelet aggregation inhibitory effect of supernates. Following chloroform:methanol extraction, platelet aggregation inhibitory activity was not observed in either the organic or aqueous phases. However, a precipitate formed at the organic/aqueous interface. Following dissolution of the precipitate in water, significant inhibitory activity was observed. Size fractionation using 10 kD and 30 kD membranes revealed significant platelet aggregation inhibition activity only in the retentate after centrifugation through the 30 kD membrane. Hence the apparent molecular weight of the inhibitor is >30 kD.

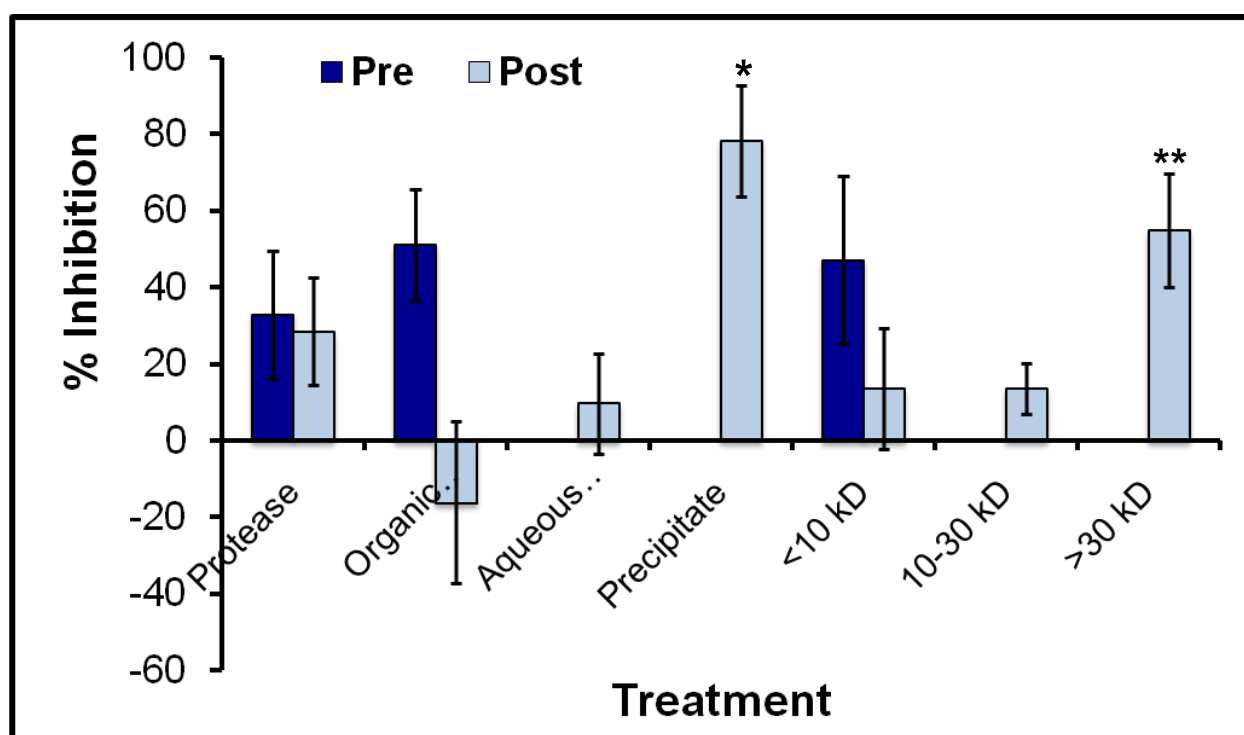


Figure 8: Characterization of the Platelet Aggregation Inhibitor.

Aggregation response to 5 μ M collagen was measured in whole blood mixed with supernates from NLR RBC stored for 42 days before ("Pre") and after ("Post") protease treatment, chloroform:methanol extraction (organic phase, aqueous phase, precipitate), and size fractionation (<10 kD, 10-30 kD, >30 kD) according to "METHODS." "% Inhibition" represents the percent decrease in the amplitude of platelet aggregation response in blood mixed with supernates compared to in blood mixed with Tyrode's buffer (* p <0.01 vs. Tyrode's buffer, n = 4; ** p <0.003 vs. Tyrode's buffer, n = 4).

4.0 DISCUSSION

Our results demonstrate that during storage, the plasma fraction of NLR RBC accumulates a substance(s) that decreases expression of GPIIb/IIIa on platelets and inhibits platelet aggregation in response to collagen. Platelet aggregation correlated with mean CD41a-FITC fluorescence, which indicates that platelet aggregation correlated with expression of GPIIb/IIIa on the platelet membrane (Figure 4). GPIIb/IIIa is the fibrinogen receptor and is constitutively expressed on platelets. In its native form, however, GPIIb/IIIa has a low binding affinity for fibrinogen. Agonists such as collagen bind to receptors on the platelet membrane and through intracellular signaling induce a change in the conformation of inactive GPIIb/IIIa to a form that binds fibrinogen avidly.[13, 14] Activated GPIIb/IIIa-fibrinogen complexes form clusters on the platelet membrane, which facilitates formation of platelet aggregates through fibrinogen bridges.[13] Thus, our data suggests that supernates inhibit platelet aggregation through reduction in the number of GPIIb/IIIa molecules on the platelet membrane.

The clinical significance of transfusion of these substances is yet to be determined, but potentially, transfusion of these substances could contribute to delayed hemostasis and increased blood loss by reduction of GPIIb/IIIa expression on platelets still in circulation. Manno et al compared the hemostatic effects of fresh whole blood (\leq 6 hours at room temperature), whole blood stored for 24-48 hours at 4-6°C, and reconstituted blood prepared from 1 unit of RBC, 1 unit FFP, and 1 unit of random donor platelets in children that underwent open heart surgery.[15] Transfusion with reconstituted blood resulted in increased blood loss compared with transfusion of either fresh whole blood or whole blood stored for 24-

48 hours at 4-6°C. Furthermore, blood loss in children transfused with the 24-48 hour stored whole blood was not statistically different from blood loss in children transfused with fresh whole blood. This suggests that endogenous rather than transfused platelets played the dominant role in establishing hemostasis since the function of platelets exposed to cold for 24-48 hours is compromised.[16] Thus, their data imply that the reconstituted blood impaired the aggregation function of the children's circulating platelets. Indeed, Manno et al reported that the 30-minute post-operation *in vitro* aggregation responses of patient platelets to ADP, epinephrine, and collagen were significantly lower in children transfused with reconstituted blood than in children transfused with either fresh or stored whole blood. In contrast, they did not find significant differences in platelet counts, prothrombin time, activated partial thromboplastin time, fibrinogen, fibrin split products, and FVIII:Rag also measured 30 minutes following surgery. Manno et al concluded that post-op coagulation tests could not explain the increased blood loss in the reconstituted group, and that lower blood losses in the fresh whole blood and stored whole blood groups was probably due to better functioning platelets. In conjunction with our results, it is plausible that substances present in the stored blood products used to make the reconstituted blood reduced the aggregation response of the children's platelets.

Platelet aggregation was inhibited equally by supernates in whole blood and PRP. This indicates that accessory blood cells such as leukocytes are not required for inhibition of platelet aggregation. Rather, the inhibitor interacts directly with the platelets to reduce the number of GPIIb/IIIa molecules expressed on the platelet membrane. This could be mediated through a receptor on the surface, which when bound by the inhibitor down regulates GPIIb/IIIa expression. Additional studies are needed to elucidate the mechanism of GPIIb/IIIa down regulation.

It is well documented that blood products accumulate proinflammatory and immunomodulatory substances during storage. Both lipids and cytokines, which are small molecular weight peptides, have been identified in the plasma fraction of stored RBC, and have been suggested to play a role in development of serious transfusion complications such as TRALI.[10, 17-21] Silliman et al demonstrated that the plasma fraction of RBC stored for at least two weeks contained lipids that primed PMNs *in vitro*, and that a PAF receptor antagonist blocked this effect.[18] They further identified several lipid species, which included lysophosphatidylcholines, that accumulated during storage and elicited the PMN-priming effect in proportion to their concentrations. In contrast to Silliman's studies on TRALI, our studies suggest that the aggregation inhibitor is not lipid since it was not extracted by the chloroform:methanol mixture. The organic phase of the chloroform:methanol mixture did not exhibit any of the aggregation inhibition activity found in the parent samples. Interestingly, activity was also not present in the aqueous phase, which suggested that the active agent was not stable to extraction with organic solvents. Indeed, a solid material was observed at the organic/aqueous phase interface. The solid material dissolved readily in water indicating a hydrophilic nature, and, once dissolved, the solution exhibited greater inhibitory activity than the original supernate.

Protease treatment did not affect the inhibitor, suggesting it is not a protein. However, its relatively large size, instability in organic solvent, and hydrophilic nature is consistent with a protein structure. There are several explanations for the observed resistance to protease treatment. One explanation is inadequate proteolytic activity or time. A second is lack of specificity, though protease from *Streptomyces griseus* has broad substrate specificity. A third explanation is that critical peptide sequences in the inhibitor may be hidden within the tertiary structure of the protein and are inaccessible to the protease. A fourth reason for resistance to proteolytic digestion is that the inhibitor is a glycoprotein. Glycoproteins are known to be more resistant to protease treatment than non-glycosylated proteins. Further studies are required to discern if the inhibitor is glycoprotein.

LR units did not contain platelet aggregation inhibitory substances, which indicates these substances either originated from leukocytes or residual platelets in the RBC units, or were generated in response to interaction of RBC membranes with products from leukocytes or residual platelets. Leukoreduction is not

required at military blood centers and because of the requirement to get RBC into the field as quickly as possible to minimize storage effects, leukoreduction is not carried out at military blood centers. Consequently, injured military personnel that require transfusions are transfused with NLR RBC, and are, therefore, transfused with RBC units that contain both, a platelet aggregation inhibitor and proinflammatory agents. Because of reported associations between age and number of units transfused and serious complications such as TRALI and MOF, early hemorrhage control is necessary to reduce the chance of development of TRALI or MOF from transfusion of added units. The development of platelet aggregation inhibitory activity in supernates of NLR RBC units during storage, however, may impair hemostasis and result in transfusion of more blood products. It is important, therefore, to conduct studies to determine if trauma patients transfused with NLR RBC require more blood products to control hemorrhage than trauma patients who are transfused with LR RBC. Information from such studies would be helpful to determine if leukoreduction should be mandatory.

5.0 CONCLUSION

We demonstrated that supernates from stored NLR packed RBC develop platelet aggregation inhibitory activity during storage. The level of inhibition increased as a function of the storage age of the RBC indicating the concentration of agent responsible for inhibition increased. The inhibitor acts directly on platelets without the need for accessory cells. Characterization data suggest the inhibitor is not lipid. The inhibitor is hydrophilic, denatured by organic solvent, and has a molecular weight greater than 30 kD. Supernates from pre-storage leukoreduced RBC did not accumulate this inhibitor during storage implicating a requirement for leukocytes and/or platelets to generate the inhibitor. Further studies are required to determine if transfusion of this material impairs hemostasis in actively bleeding individuals such that transfusion requirements are increased.

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7.0 REFERENCES

- [1] Perkins J, Cap A, Weiss B, Reid T, Bolan C: Massive transfusion and nonsurgical hemostatic agents. *Critical Care Medicine*. 2008;**36**: S325-S39.
- [2] Alam H, Burris D, DaCorta J, Rhee P: Hemorrhage control in the battlefield: role of new hemostatic agents. *Military Medicine*. 2005;**170**: 63-9.
- [3] Kelly J, Ritenour A, McLaughlin D, Bagg K, Apodaca A, Mallak C, Pearse L, Lanwick M, Champion H, Wade C, Holcomb J: Injury severity and causes of death from operation iraqi freedom and operation enduring freedom: 2003-2004 versus 2006. *Journal of Trauma*. 2008;**64**: S21-S7.
- [4] Spahn DR, Rossaint R: Coagulopathy and blood component transfusion in trauma. *British Journal of Anaesthesiology*. 2005;**95**: 130-9.
- [5] Bellamy R, Maningas P, Vayer J: Epidemiology of trauma: Military experience. *Annals of Emergency Medicine*. 1986;**15**: 1384-8.

- [6] Holcomb JB, Jenkins D, Rhee P, Johannigman J, Mahoney P, Mehta S, Cox ED, Gehrke MJ, Belman GJ, Schreiber M, Flaherty SF, Grathwohl KW, Spinella PC, Perkins JG, Beekley AC, Schwab CW, Moore FA, Champion HR, Hoyt DB, Hess JR: Damage control resuscitation: Directly addressing the early coagulopathy of trauma. *Journal of Trauma*. 2007;**62**: 307-10.
- [7] Davenport R, Karim B: Coagulopathy in trauma patients: importance of thrombocyte function? *Current Opinion in Anaesthesiology*. 2009;**22**: 261-6.
- [8] Hardy J, Samama M: Massive transfusion and coagulopathy. *Transfusion Alternatives in Transfusion Medicine*. 2003;**4**: 199-210.
- [9] Hess JR: Blood and coagulation support in trauma care. *Hematology*. 2007;**2007**: 187-91.
- [10] McFaul SJ, Corley JB, Mester CW, Nath J: Packed Blood Cells Stored in AS-5 Become Proinflammatory During Storage. *Transfusion*. 2009;**49**: 1451-60.
- [11] Matheu FA, McFaul SJ: Supernates from stored red blood cells inhibit platelet aggregation. *Transfusion*. 2010.DOI: 10.1111/j.1537-2995.2010.02582.x
- [12] Bligh E, Dyer W: A rapid method of total lipid extraction and purification. *Canadian Journal of Physiology and Pharmacology*. 1959;**37**: 911-7.
- [13] Levy-Toledano S: Platelet signal transduction pathways: Could we organize them into a 'hierarchy'? *Haemostasis*. 1999;**29**: 4-15.
- [14] Naimushin YA, Mazurov AV: Role of glycoprotein IIb-IIIa (α IIb β 3 integrin) in stimulation of secretion from platelet granules. *Biochemistry (Moscow)*. 2003;**68**: 209-16.
- [15] Manno C, Hedberg K, Kim H, Bunin G, Nicolson S, Jobes D, Schwartz E, Norwood W: Comparison of the hemostatic effects of fresh whole blood, stored whole blood, and components after open heart surgery in children. *Blood*. 1991;**77**: 930-6.
- [16] Murphy S, Gardner FH: Platelet preservation: Effect of storage temperature on maintenance of platelet viability – Deleterious effect of refrigerated storage. *New England Journal of Medicine*. 1969;**280**: 1094-8.
- [17] Koch CG, Li L, Sessler DI, Figueroa P, Hoeltge GA, Mihaljevic T, Blackstone EH: Duration of red-cell storage and complications after cardiac surgery. *The New England Journal of Medicine*. 2008;**358**: 1229-39.
- [18] Siliman CC, Clay KL, Thurman GW, Johnson CA, Ambruso DR: Partial characterization of lipids that develop during the routine storage of blood and prime the neutrophil NADPH oxidase. *Journal of Laboratory and Clinical Medicine*. 1994;**124**: 684-94.
- [19] Sparrow RL, Patton KA: Supernatant from stored red blood cell primes inflammatory cells: influence of prestorage white cell reduction. *Transfusion*. 2004;**44**: 722-30.
- [20] Zallen G, Moore EE, Ciesla DJ, Brown M, Biffl WL, Siliman CC: Stored red blood cells selectively activate human neutrophils to release IL-8 and secretory PLA. *Shock*. 2000;**13**: 29-33.
- [21] Zallen G, Offner PJ, Moore EE, Blackwell J, Ciesla DJ, Gabriel J, Denny C, Siliman CC: Age of transfused blood is an independent risk factor for postinjury multiple organ failure. *American Journal of Surgery*. 1999;**178**: 570-2.

